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A Unified Radiometric Assay System for the Gaba-Glutamate Regulating Enzymes

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A UNIFIED RADIOMETRIC ASSAY SYSTEM FOR THE GABA-GLUTAMATE REGULATING ENZYMES

by

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A thesis submitted in partial fulfillment
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of

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ABSTRACT

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the GABA-glutamate Regulating Enzymes

by

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Utah State University, 1978

Major Professor: Dr. Elizabeth A. Boeker
Department: Chemistry and Biochemistry

The purpose of this paper was to develop a single assay system for the enzymes which regulate GABA and glutamate concentrations in brain and nerve tissue. Since all the enzymes produce L-glutamate, their activities were measured by coupling them to L-glutamate decarboxylase. Enzymatic activity was determined by measuring the release of CO_2 from radioactive substrates. The glutamate decarboxylase was obtained from a commercial acetone powder by simplifying existing procedures. The glutamate decarboxylase produced was of sufficient purity to be used in the coupled assays, which were checked with commercial preparations of each enzyme, where available, and with crude brain homogenates. All of the assays were shown to be linear with respect to both time and enzyme concentration, thus assuring the feasibility of the technique.

(69 pages)

INTRODUCTION

Glucose metabolism in the brain has been studied with labelled glucose, pyruvate, and γ-aminobutyric acid (GABA). It has been shown that this metabolic pathway produces significant amounts of glutamic acid and GABA (Beloff-Chain, A., 1955; Gaitonde, M.K., 1965; and Balazs, R., 1966). Glutamate and GABA are present in brain and nerve tissue on the order of 10 mM and 1 mM respectively (Awapara, J., 1950 and Roberts, E., 1950). Because of this unusually high concentration in brain and nerve tissue, a role for glutamate and GABA in nerve impulse transmission has been sought.

It has been established that very small amounts of GABA inhibit depolarization of nerve cell membranes and it is thought, though it is still uncertain, that glutamate plays a role in the stimulation of the membrane (Werman, R., 1966 and Krnjevic, K., 1974). Knowledge of the enzymes which control the cellular concentrations of GABA and glutamate may help to understand the control of the nerve impulse.

These enzymes are 1, glutamate dehydrogenase, 2, glutamate decarboxylase, 3, glutaminase, 4, glutamine synthetase, 5, glutamate-oxaloacetate transaminase, 6, glutamate-pyruvate transaminase, and 7, GABA-α-ketoglutarate transaminase (Figure 1).

FIGURE 1. The enzymatic reations catalyzed by the seven enzymes which regulate glutamate and GABA metabolism in the brain. 1) glutamate dehydrogenase, 2) L-glutamate decarboxylase, 3) L-glutaminase, 4) L-glutamine synthetase, 5) L-glutamate-oxaloacetate transaminase, 6) L-glutamate-pyruvate transaminase, and 7) GABA-α-ketoglutarate transaminase.

1. α-ketoglutarate + NH_4+ + NADH $\rightleftharpoons$ L-glutamate + NAD^+

2. L-glutamate $\longrightarrow$ GABA + CO_2

3. L-glutamine + H_2O $\longrightarrow$ L-glutamate + NH_4+

4. L-glutamate + NH_4+ + ATP $\longrightarrow$ L-glutamine + ADP + Pi

5. α-ketoglutarate + L-aspartate $\rightleftharpoons$ L-glutamate + oxaloacetate

6. α-ketoglutarate + L-alanine $\rightleftharpoons$ L-glutamate + pyruvate

7. α-ketoglutarate + GABA $\rightleftharpoons$ L-glutamate + succinic semialdehyde

This thesis is an attempt to lay the groundwork for the larger goal of understanding the regulation of GABA and glutamate concentrations in brain and nerve tissue. This work intends to establish a convenient, accurate, and sensitive set of assays for the enzymes controlling GABA and glutamate metabolism, using a similar technique in each case. The assays use radioactively labelled substrates to produce the sensitivity needed to measure enzyme levels in crude tissue extracts, providing a sensitivity lacking in most commonly used spectrophotometric assays. Since all of the enzymes have a common substrate, L-glutamate, it was decided to couple each of them to glutamate decarboxylase from Escherichia coli, thus unifying the assay techniques. This greatly simplifies routine experimental measurements of the glutamate-GABA system.

Since no commercial preparation of glutamate decarboxylase of sufficient purity exists for these assays, the enzyme has been obtained from a readily available, inexpensive commercial source of low purity by simplifying the purification of Shukuya and Schwert (I. 1960).

After purification, the glutamate decarboxylase was used to assay six of the seven glutamate regulating enzymes in a coupled reaction. The enzyme assays were set up and tested with commercial preparations of each enzyme, where available, and then checked with crude brain homogenates to assure feasibility. No attempt has been made to assay

glutamine synthetase with this system, since it would be necessary to measure the disappearance of glutamate; a better assay already exists (Prusiner, S., and Milner, L., 1970).

LITERATURE REVIEW

The idea of using specific amino acid decarboxylases as an accurate and convenient method for the quantitative measurement of amino acids is not a new one. Umbreit and Gunsalus (1945) proposed using extracts of E. coli strains known to be high in certain amino acid decarboxylases for this purpose. Taylor and Gale (1945) proposed the use of Clostridium welchii strain SR12 preparations for the assay of glutamic acid. Since this organism also contains an active glutaminase and an aspartic acid decarboxylase, it is useless for work in an unknown mixture of amino acids or in biological fluids. Obviously, a preparation high in one particular decarboxylase activity but very low in others was needed.

Nine years later, Najjar and Fisher (1954) reported the preparation of a purer bacterial amino acid decarboxylase. They purified glutamate decarboxylase more than eight-fold from E. coli type ATCC 11246, a strain isolated in their laboratory. They reported their enzyme to be "a highly active and specific L-glutamic acid decarboxylase free from any other amino acid decarboxylase." They claimed that it contained no glutamic acid racemase or glutaminase activity, thereby allowing the assay of L-glutamic acid in the presence of glutamine and other amino acids. No

estimate of the homogeneity of this enzyme preparation was made.

In a series of papers, Shukuya and Schwert (I., II., III., 1960) described a detailed procedure for the purification of glutamate decarboxylase from E. coli strain 26, producing a higher state of purity than had previously been achieved. In these papers they also described many of the physical characteristics of their purified enzyme, stating that: "From ultracentrifugal and electrophoretic analyses the preparation appears to be approximately 90% homogeneous. The molecular weight, estimated from sedimentation velocity and diffusion measurements, is 300,000. The pH optimum is 3.8. The Michaelis constant for glutamate is 0.82 mM in pyridine-pyridine hydrochloride buffer, pH 4.6 at 36°C. The enzyme is activated by pyridoxal phosphate and by chloride ion and is inhibited by acetate." They also claim that the enzyme rapidly loses activity in dilute solution at neutral pH and 0°C. They report a specific activity from 41-73 μmoles/minute/mg protein in the presence of added pyridoxal-P. No mention was made as to whether the remaining 10% impurity contained any other amino acid decarboxylase activity. This scheme has been the basis for all further purifications of this enzyme, except for that of Strausbauch and Fischer (1967).

Huntley and Metzler (1967) modified the cell growth conditions and the method of cellular disruption and

obtained an enzyme preparation with a "specific activity approximately the same as that found by Shukuya and Schwert." Fonda (1971) isolated glutamate decarboxylase from E. coli strain ATCC 11246, by using the procedures of both Shukuya and Schwert (1960) and Huntley and Metzler (1967). Her main modification was the addition of 0.1 mM pyridoxal-P and 1 mM dithiothreitol to all buffers. This preparation had a specific activity of 76 μmoles/minute/mg.

Strausbauch and Fischer (1967) describe the purification, crystallization and some of the properties of glutamate decarboxylase from E. coli strain W. The enzyme was obtained as a by-product of the purification of pyruvate oxidase. This crystalline enzyme has the highest specific activity reported to date; 120 μmoles/minute/mg at pH 4.5 and 38°C. The K_M for glutamate is 0.54 mM. The three-times crystallized enzyme gives a single band on disc gel electrophoresis at pH 7.9. In a second paper, Strausbauch and Fischer (1970) reported that sedimentation equilibrium experiments in guanidine-HCl and gel electrophoresis in sodium dodecyl sulfate gave molecular weights of 50,000 for the denatured enzyme subunit, indicating that the native enzyme is a hexamer. They also obtained a value for the absorbance index of the crystalline enzyme of $A_{280}^{1\%} = 17.0$ at pH 7.0. Because the crystallization process is difficult and the yield from this procedure is

low, this method was not used to obtain glutamate decarboxylase. Instead, an attempt was made to simplify the method of Shukuya and Schwert.

There are several methods used for the assay of glutamate decarboxylase. The first is the conventional Warburg manometric technique in which the amount of CO_2 evolved from the reaction mixture is related to the enzymatic activity. This was the method used by most of the initial investigators and is still in use today. Zeman and coworkers (1973) have recently described an automated assay for the enzyme based on this procedure. But, because of the large quantity of enzyme needed to produce measureable amounts of CO_2, and because other CO_2 producing systems can be present in crude cell extracts, this method is no longer widely used.

In a second method, a spectrophotometric assay, the GABA formed during decarboxylation is measured by the "Gabase system," in which GABA transaminase is coupled to succinic semialdehyde dehydrogenase (Scott, E., and Jakoby, W., 1959). The reactions catalyzed are:

$$\text{GABA} + \alpha\text{-ketoglutarate} \rightleftharpoons \text{SSA} + \text{glutamate}$$

$$\text{SSA} + \text{NAD(P)}^+ + \text{H}_2\text{O} \rightleftharpoons \text{succinate} + \text{NAD(P)H}$$

The velocity of the reaction is then estimated from the rate of reduction of NAD(P)^+. Again, this type of assay is somewhat insensitive; it is also not well suited to the performance of large numbers of assays.

The third type of assay, which is most sensitive, is a modification of the Warburg technique in which radioactively labelled substrate is used. The $^{14}CO_2$ formed is released by acidification, trapped in an absorbing medium, and counted in a liquid scintillation counter. This method was used throughout this work because of its inherent sensitivity and the fact that even if extraneous CO_2 is formed, it is not measured. A full description of the actual assay procedure will be presented later.

There have been several methods used for the determination of glutaminase activity. The first was based on the determination of free ammonia with Nessler's reagent after microdistillation of the reaction mixture, as described by Meister (1955) and Horowitz (1968). More recently, Huang (1974) describes a method in which the ammonia is measured directly by an ammonia electrode. The other commonly employed assay methods for glutaminase involve paper and thin layer chromatographic separation of glutamate from glutamine with colorimetric determination of glutamate (Wood, A., 1972; Curthoys, N., 1973). Another popular assay is to couple the reaction to glutamate dehydrogenase and follow the rate of reduction of NAD^+. Prusiner and Milner (1970) have published a sensitive assay technique based on the separation of ^{14}C-labelled glutamine from glutamate on an ion exchange column.

Glutamate dehydrogenase activity has been measured principally by two methods. The first method (Frieden, C., 1963), is based upon the reduction of NAD^+ or the oxidation of NADH measured at 340 nm. The second method uses ^{14}C-α-ketoglutarate as a substrate and the labelled glutamic acid produced is separated from the substrate on an ion exchange column and counted in a liquid scintillation counter. This is the most sensitive assay, but it is not convenient for repetitive assays.

The remaining three enzymes to be discussed are all transaminases and have similar assay systems. Since all produce glutamate, each can be coupled to the glutamate dehydrogenase system discussed above. Glutamate-oxaloacetate transaminase has ordinarily been assayed spectrophotometrically by coupling it to malate dehydrogenase and following the rate of oxidation of NADH at 340 nm (Karmen, A., 1955; Amador, E., 1962; and Sizer, I., 1962). Jenkins (1959) measured the oxaloacetate formed in the transamination reaction spectrophotometrically at 280 nm. Under the assay conditions, the light absorbed is proportional to the amount of oxaloacetate produced. Itoh and Srere (1970) have described a new assay for glutamate-oxaloacetate transaminase in which the oxaloacetate formed is measured by coupling the reaction to that of citrate synthetase. The CoASH produced is then reacted with DTNB to produce thionitrobenzoate, which absorbs at 412 nm.

Glutamate-pyruvate transaminase has mainly been assayed with lactate dehydrogenase and NADH in a system similar to that described for the glutamate-oxaloacetate transaminase described above. This method was published by Grein and Pfleiderer (1958) and later modified by Segal and Hopper (1962). A colorimetric assay for glutamate-pyruvate transaminase takes advantage of the fact that pyruvate forms a red color when incubated with salicylaldehyde in strongly alkaline solution (Lenard, P., and Straub, F. B., 1942). This procedure has also been used by Saier (1967) and Jenkins (1970).

The glutamate formed by GABA-α-ketoglutarate transaminase can be assayed colorimetrically using a copper chelating reaction, as described by Baxter and Roberts (1958). The only other assay for GABA-α-ketoglutarate transaminase that has not already been described, at least in principle, is one proposed by Sytinsky and Vasilijev (1969). This is a colorimetric assay based on the reaction of 3-methyl-2-benzthiazolone-2-hydrazone with the succinic semialdehyde formed during the enzymatic reaction. The concentration of SSA in the samples was determined by using a calibration curve at 660 nm.

In conclusion, it appears that a number of assays are available for these enzymes, most of them based on rather different principles. Some are accurate and reliable; some are not. Some are convenient and sensitive; others are

just the opposite. Using the best of these for each enzyme that must be assayed, in a study of the regulation of GABA and glutamate metabolism in brain and nerve tissue, would result in a system of four or five different types of assays that, at best, would be tedious, difficult and confusing. Clearly a set of simple, sensitive assays based on a single principle and employing similar techniques would simplify matters considerably.

EXPERIMENTAL PROCEDURE

Materials

Diethylaminoethyl cellulose (DE-52) was purchased from the Whatman Company. Uniformly labelled L-(^{14}C)-glutamine, L-(^{14}C)-glutamate, and α-(1-^{14}C)-ketoglutarate were obtained from New England Nuclear. Enzyme grade ammonium sulfate was purchased from Schwarz-Mann. An acetone powder of E. coli containing glutamate decarboxylase activity was purchased from Sigma Chemical Company. Phenylmethylsulfonylfluoride (PMSF), protamine sulfate, pyridoxal-P, dithiothreitol, and all other enzymes, substrates and cofactors were obtained from Sigma.

Activity Assays

Glutamate decarboxylase was routinely assayed by a modification of the method of Morris and Pardee (1965) in which radioactive CO_2 is released from L-(^{14}C)-glutamate. The standard assay mixture contained 60 µmoles of pyridine HCl buffer, pH 4.5, 3.0 µmoles of L-(^{14}C)-glutamate (5.2×10^4 D.P.M./µmole), 0.03 µmoles of pyridoxal-P and an appropriate amount of enzyme in a total volume of 0.3 ml. The reaction was started by the addition of enzyme. The incubations were carried out at 37°C in a stoppered tube which held a fluted piece of filter paper near the

top. The paper was spotted with 0.05 ml of an ethanolamine: methylcellosolve (2:1) solution. At the end of the incubation the reaction was stopped with 0.1 ml of 50%(w/v) trichloroacetic acid. The stoppered tube was then incubated for an additional 30 minutes at 37°C, in order to transfer all the $^{14}CO_2$ evolved to the filter paper. The paper was removed, placed in 10 ml of scintillation fluid and counted in a Beckman Scintillation Counter.

Under these conditions, the evolution of CO_2 is proportional to the amount of enzyme added; the response is linear until 30% of the substrate has been consumed. One unit of glutamate decarboxylase represents the amount of enzyme catalyzing the release of 1 μmole of CO_2/minute.

The coupled enzyme assays were set up so that the initial enzyme reaction produced glutamic acid. α-(1-^{14}C)-ketoglutarate was used as the substrate in all assays except for glutaminase, where L-(^{14}C)-glutamine was used. The specific conditions of each assay are given in Tables 1-6. After a 15 minute incubation period, the assay reaction was stopped by adding enough 1 N HCl to reduce the pH to 4.5. Pyridine-HCl buffer, pH 4.5, and pyridoxal-P were then added to final concentrations of 0.2 M and 0.1 mM, respectively. Glutamate decarboxylase was added to initiate the second reaction. The final total volume was 0.75 ml. The procedure described above for the glutamate decarboxylase reaction alone was then followed exactly.

Table 1. Specific Conditions for Glutamate Dehydrogenase Assay

	Stock Solution	µl used per assay	Final Assay Concentration, mM
1.	40 mM α-ketoglutarate, 1-^{14}C(8×10^3 DPM/µmole) 40 mM NH_4Cl 0.2 M Na_2HPO_4, pH 7.7	200	20 20 100
2.	80 mM NADH	100	20
3.	50 mM Na_2HPO_4, pH 7.66, plus enzyme	100	0-12
	Coupled Glutamate Decarboxylase Reagents		
1.	1 N HCl	50	-
2.	1.5 M Pyridine-HCl, pH 4.5	175	200
3.	0.75 mM Pyridoxal-P	100	0.1
4.	Glutamate Decarboxylase, 44 µmoles/min/mg	2.0 µmoles/min	-

Table 2. Specific Conditions for Glutamic-Oxaloacetic Transaminase Assay

	Stock Solution	µl used per assay	Final Assay Concentration, mM
1.	80 mM α-ketoglutaric acid, 1-^{14}C(10^4 DPM/µmole) 80 mM L-aspartate 0.2 M Na_2HPO_4, pH 7.7	200	40 40 100
2.	0.75 mM Pyridoxal-P	50	0.1
3.	50 mM Na_2HPO_4, pH 7.7, plus enzyme	150	-
	Coupled Glutamate Decarboxylase Reagents		
1.	1 N HCl	60	-
2.	1.5 M Pyridine-HCl, pH 4.5	150	200
3.	0.75 mM Pyridoxal-P	100	0.1
4.	Glutamate Decarboxylase, 44 µmoles/min/mg	3.0 µmoles/min	-

Table 3. Specific Conditions for Glutaminase Assay

Stock Solution		µl used per assay	Final Assay Concentration, mM
1.	40 mM L-glutamine, U-^{14}C(5×10^4 DPM/µmole) 0.3 M K_2HPO_4, pH 8.0 0.4 mM EDTA	200	20 150 0.2
2.	10 mM Borate, pH 8.0 0.2 mM EDTA Enzyme	200	-
	Coupled Glutamate Decarboxylase Reagents		
1.	1 N HCl	50	-
2.	1.5 M Pyridine-HCl, pH 4.5	175	200
3.	0.75 mM Pyridoxal-P	100	0.1
4.	Glutamate Decarboxylase, 44 µmoles/min/mg	2.0 µmoles/min	-

Table 4. Specific Conditions for Glutamate-Pyruvate Transaminase Assay

	Stock Solution	µl used per assay	Final Assay Concentration, mM
1.	8 mM α-ketoglutarate, 1-^{14}C(3×10^4 DPM/µmole) 60 mM L-alanine 0.2 M Tris, pH 8.1	200	4 30 100
2.	0.8 mM Pyridoxal-P	50	0.1
3.	50 mM Tris, pH 8.1, plus enzyme	150	-
	Coupled Glutamate Decarboxylase Reagents		
1.	1 N HCL	30	-
2.	1.5 M Pyridine-HCl, pH 4.5	200	200
3.	0.75 mM Pyridoxal-P	100	0.1
4.	Glutamate Decarboxylase, 44 µmoles/min/mg	1.5 µmoles/min	-

Table 5. Specific Conditions for GABA-α-ketoglutarate Transaminase Assay

	Stock Solution	µl used per assay	Final Assay Concentration, mM
1.	80 mM α-ketoglutarate, 1-^{14}C(1.2x10^{4} DPM/µmole) 80 mM GABA 0.2 M Tris, pH 8.2	200	40 40 100
2.	0.8 mM Pyridoxal-P	50	0.1
3.	50 mM Tris, pH 8.2, plus enzyme	150	-
	Coupled Glutamate Decarboxylase Reagents		
1.	1 N HCl	40	-
2.	1.5 M Pyridine-HCl, pH 4.5	170	200
3.	0.75 mM Pyridoxal-P	100	0.1
4.	Glutamate Decarboxylase, 44 µmoles/min/mg	3.0 µmoles/min	-

Table 6. Specific Conditions for Glutamate Decarboxylase Assay

Stock Solution	µl used per assay	Final Assay Concentration, mM
1. 30 mM L-glutamate, U-^{14}C(5×10^4 DPM/µmole) 0.6 M Pyridine-HCl, pH 4.5	100	10 200
2. 0.3 mM Pyridoxal-P	100	0.1
3. 0.2 M Pyridine-HCl, pH 4.5 plus Glutamate Decarboxylase	100	-

Protein Determination

Protein concentration was determined by the method of Lowry et al. (1951) on impure preparations (specific activity less than 40 units/mg), and spectrophotometrically on more highly purified material, using an absorbance index $A_{280}^{1\%}$ of 17.0 at pH 7 (Strausbauch and Fischer, 1970). All measurements were made on a Beckman DU Spectrophotometer.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate was carried out at 8 mA/tube according to Davis et al. (1967) in 7.5% gels. Samples were pretreated at 70°C for 15 minutes in 1% dodecyl sulfate (Sabo, et al., 1974). 20 μg of protein was applied for purity checks; gels were stained for 2 hours with Coomassie Brilliant Blue. Gels were destained electrophoretically and stored in a solution containing 7.5% acetic acid and 5% methanol.

Purification of Enzyme

The procedure presented here for the purification of glutamate decarboxylase is based on that of Shukuya and Schwert (1960). A 6% suspension of *E. coli* acetone powder in 50 mM sodium phosphate (NaPi), pH 6.0, with 2 mM PMSF was allowed to autolyze for eight hours at 25°C. This suspension is made by dissolving 7 mg PMSF/ml of 50% ethanol

and adding 0.5 ml of this solution to each 10 ml of buffer. The PMSF solution and 50 mM NaPi are mixed at 37°C and allowed to cool to about 30°C. The acetone powder is added and the mixture is homogenized in a tissue grinder. The pH of the suspension after autolysis was about 6.5. The cell debris was collected at 27,300 x g at 4°C for 60 minutes and discarded.

A 2% solution of protamine sulfate, containing 2 mM PMSF, all at pH 6.0, was added slowly, with stirring, to the crude extract at 25°C, until the heavy precipitate stopped forming. The precipitate was removed by centrifugation and discarded. This and all later centrifugations were carried out at 27,300 x g for 30 minutes at 4°C.

The supernatant from the protamine sulfate step was brought to 40% saturation with ammonium sulfate by adding the solid salt, very slowly, with stirring. The precipitate was collected by centrifugation, dissolved in distilled water, checked for activity and discarded. The supernatant was then brought to 60% saturation with ammonium sulfate as before. The precipitate was collected and dissolved in distilled water to a concentration of 35 mg protein/ml.

The ammonium sulfate concentration in the enzyme solution was reduced by a 12 hour dialysis against 0.1 M pyridine-HCl pH 4.7, containing 0.1 mM pyridoxal-P and 1 mM dithiothreitol. The resulting enzyme solution was dialyzed

for 12 hours against several changes of 50 mM NaPi pH 6.0, containing 0.1 mM pyridoxal-P and 1 mM dithiothreitol. The precipitate was removed by centrifugation.

The glutamate decarboxylase was applied to a 2.6 x 25 cm DEAE-cellulose column which had been equilibrated with 50 mM NaPi, pH 6.0. The column was washed with the same buffer until the A_{280} was less than 0.05. Twenty ml fractions were then collected at a flow rate of 50 ml/hour at 10°C and glutamate decarboxylase was eluted with an 800 ml linear gradient between 50 and 250 mM NaPi, pH 6.0. Fractions with a specific activity of 50 or more were pooled and concentrated in an Amicon ultrafiltration cell using an XM-50 membrane.

This enzyme solution was dialyzed against 0.1 M pyridine-HCl, pH 4.5, containing 0.1 mM pyridoxal-P and 1 mM dithiothreitol. The resulting precipitate was discarded. The supernatant was allowed to stand for one week in the pyridine buffer until all precipation stopped, and was then dialyzed against 0.1 M pyridine-HCl pH 5.5. Purified glutamate decarboxylase could be stored at 5° for two months without loss of activity.

RESULTS AND DISCUSSION

Glutamate Decarboxylase Purification

A typical elution diagram for glutamate decarboxylase is shown in figure 2. 50 mM NaPi, pH 6.0, was used to wash the column before the gradient was applied. This removed a substantial amount of contaminating protein, some of which probably eluted with glutamate decarboxylase when a gradient was applied directly, as in the method of Shukuya and Schwert (I., 1960). The activity peak resulting from the elution of glutamate decarboxylase from the column with the NaPi gradient seems unusually broad. Smaller gradient volumes might help to reduce this broadening. The specific activity is quite constant across the peak of glutamate decarboxylase activity, suggesting that the preparation is quite pure.

Table 7 summarizes a typical purification procedure for glutamate decarboxylase from 16 grams of *E. coli* acetone powder. The final specific activity was routinely between 43 and 72 units/mg. This number corresponds well with those obtained by Shukuya and Schwert (I., 1960), Huntley and Metzler (1967), and Fonda (1970), who obtained preparations with specific activities between 41 and 77 units/mg.

Figure 2. Chromatography of 500 mg of crude glutamate decarboxylase on DEAE cellulose. Glutamate decarboxylase was eluted with a gradient of NaPi, pH 6.0, from 50 mM to 250 mM. Other conditions are specified in the text.

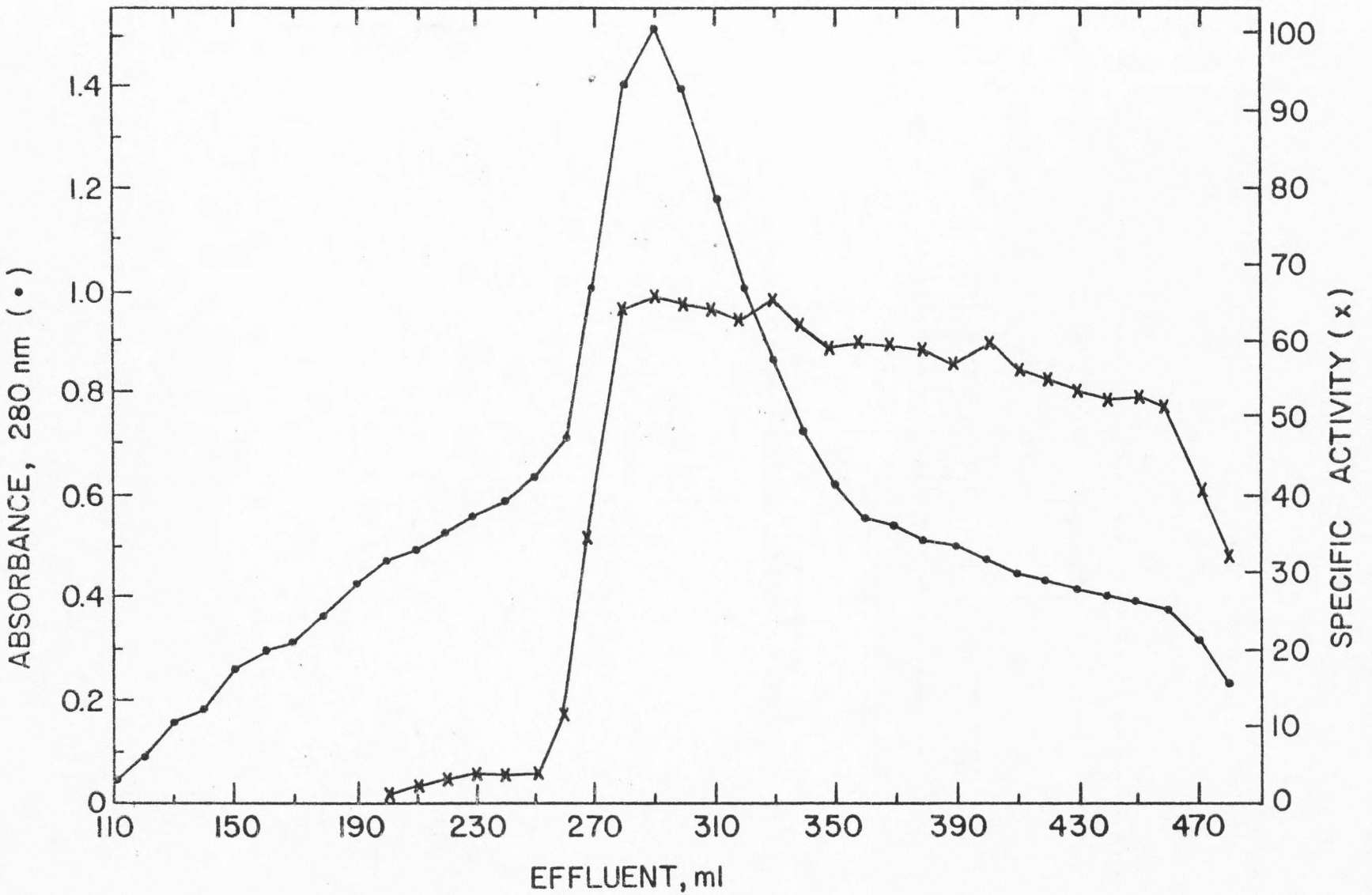

ABSORBANCE, 280 nm (•)
SPECIFIC ACTIVITY (x)
EFFLUENT, ml
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
0
10
20
30
40
50
60
70
80
90
100
110
150
190
230
270
310
350
390
430
470

Table 7. Summary of Glutamate Decarboxylase Purification

Step	Total Volume (ml)	Total Protein (mg)	Sp Act (units/mg)	Total Units	% Recovery
Crude Extract	214	2,700	5.3	14,300	100
Protamine Sulfate Supernatant	240	2,200	6.4	14,000	99
40-60% Saturated Ammonium Sulfate Ppt.	25	860	15.0	12,900	91
Post-Dialysis Supernatant I	28	500	22.0	11,000	78
DEAE-Cellulose Chromatography	30	100	60.0	6,000	42
Post-Dialysis Supernatant II	36	83	71.0	5,900	41

Purity of Glutamate Decarboxylase

The final enzyme preparation did not decarboxylate glutamine or α-ketoglutarate. The conditions used were such that an activity of 0.05 units/mg would easily have been detectable. This was a requirement for its use in coupled enzyme assays, since these two substrates would be radioactively labelled and any CO_2 originating from them would be detected. The preparation also showed less than 0.1% activity toward lysine and aspartic acid, indicating that it was not contaminated by these decarboxylases.

Polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate yielded one major band and one fast migrating species that appeared to be less than 1% of the intensity of the major band (Figure 3). Polyacrylamide disc gel electrophoresis gave little useful information about the purity of the preparation because, at the pH of these gels (above 7.5), glutamate decarboxylase yields many bands which are all enzymatically active. This was shown by slicing an unstained gel in accordance with known band patterns from previously stained gels, dispersing the gel slices with a glass rod in the assay buffer, and assaying for glutamate decarboxylase as before (Sabo, et al., 1974). This experiment seems to indicate that it is an associating-disassociating system in this pH range.

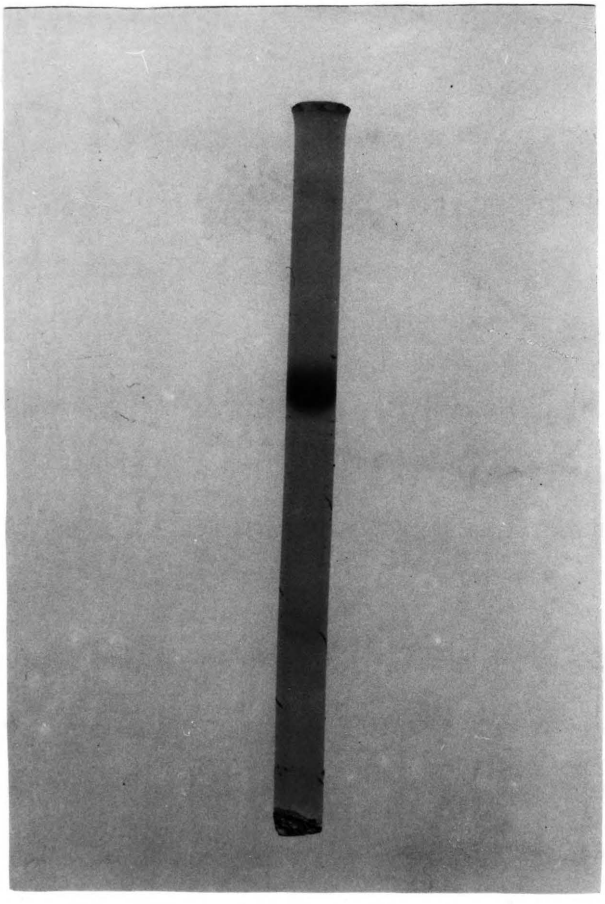


Figure 3. Polyacrylamide gel electrophoresis of glutamate decarboxylase in 0.1% sodium dodecyl sulfate. Approximately 20 μg of protein was applied under the conditions described in the text.

One of the original aims of this project was to obtain a preparation of glutamate decarboxylase of high purity, by simplifying the method of Shukuya and Schwert (I., 1960). Although the procedure developed here does not eliminate many steps, the changes that were made improve the overall reproducibility of the procedure.

The first change is in the extraction of the acetone powder. Shukuya and Schwert extracted a water solution of

the acetone powder for 24 to 48 hours while maintaining the pH between 6.0 and 6.5 by periodically adding 0.1 N NaOH. Since the addition of this solution of NaOH is tedious and sometimes causes localized precipitation and loss of enzyme activity, 50 mM NaPi, pH 6.0, was used to maintain the pH within the required range. In addition, it was found that the enzyme activity reached a maximum after only eight hours of extraction. This shortened time period reduced the opportunity for proteolytic degradation to occur. PMSF was also added to inhibit proteases.

The protamine sulfate precipitation of nucleic acids also destroyed glutamate decarboxylase activity in each of the initial attempts. It was then discovered that the addition of an unbuffered solution of protamine sulfate to the unbuffered extract reduced the pH of the solution to less than 3.0, destroying all activity. Again, the extraction of the acetone powder in 50 mM NaPi helps to insure the success of the following steps.

Since the heat step and the ammonium sulfate fractionation caused too great a loss of activity for the amount of purification produced, the first was eliminated and the second was refined. Changing the ammonium sulfate fractionation limits to 40 to 60% improved both the purity and the yield of the resulting enzyme.

The pyridine dialysis at pH 4.5, used by Shukuya and Schwert after the ammonium sulfate step, was found to cause

excessive co-precipitation of glutamate decarboxylase. This was eliminated by dialysing at pH 4.7 and using an initial protein concentration of 35 mg/ml. The pH 4.5 dialysis proved useful at a later step in the purification, after the DEAE-cellulose chromatography. The only other change in the existing procedure was the 50 mM NaPi wash of the DEAE column, as discussed above.

In conclusion, it appears that the procedure presented here for the purification of glutamate decarboxylase from an inexpensive commercial source is both simpler and more reproducible than previous procedures.

Coupled Enzymatic Assays

The glutamate decarboxylase obtained by the method just described was sufficiently pure to be used in coupled assays of the six enzymes of the GABA-glutamate system.

All of the assays were linear with respect to time and enzyme concentration, with both the commercial enzyme preparations and the crude brain homogenates. All the assays were reproducible, and only the short time samples (less than 15 minutes) showed any deviation from linearity. This problem was overcome by running these samples separately from the rest of the group and carefully controlling their incubation times. These pipetting "lag" times were unimportant in the timed assays over 15 minutes.

Table 8 shows the amount of substrate used per assay and the amount of substrate that can be consumed before the reaction becomes non-linear.

Figures 4 to 6 show the results of the coupled assays for glutamate dehydrogenase. The commercial enzyme used was an ammonium sulfate suspension, isolated from beef liver, that had a reported specific activity of 40-60 μmoles/min/mg. The assay system was also checked with glutamate dehydrogenase which was extracted from frozen beef brain.

Figures 7 and 8 show the results of the coupled assays for glutaminase. Since levels of glutaminase were known to be high in the brain, the assay system was checked with only extracts of frozen beef brain (Svenneby, 1971).

Figures 9 to 11 show the results of the coupled assays for glutamate-oxaloacetate transaminase. The commercial enzyme used was an ammonium sulfate suspension, isolated from pig heart, that had a reported specific activity of 200 μmoles/min/mg. The assay system was also checked with extracts from frozen beef brain and was found to behave in a similar manner.

Figures 12 to 15 show the results of the coupled assays for glutamate-pyruvate transaminase. The commercial enzyme used was an ammonium sulfate suspension, isolated from pig heart, that had a reported specific activity of 80 μmoles/min/mg. The coupled assays were also done with

Table 8. Additional Assay Specifications for the GABA-Glutamate Enzymes

Enzyme	Substrate	Reaction Volume (ml)	μmoles substrate per assay	% Substrate Consumed Before Linearity Deviation
Glutamate Dehydrogenase	1-(^{14}C)-α-ketoglutarate	0.4	8	45
Glutamate-Oxaloacetate Transaminase	1-(^{14}C)-α-ketoglutarate	0.4	16	30
Glutamate-Pyruvate Transaminase	1-(^{14}C)-α-ketoglutarate	0.4	1.6	50
Glutaminase	U-(^{14}C) glutamine	0.4	8	40
GABA-α-Ketoglutarate Transaminase	1-(^{14}C)-α-ketoglutarate	0.4	16	40

Figure 4. Assay of commercial beef liver glutamate dehydrogenase using the coupled glutamate decarboxylase system. Protein concentration was 0.2 mg/ml. Time of assay was 15 minutes. All points on this and all following graphs represent the average of at least 3 runs.

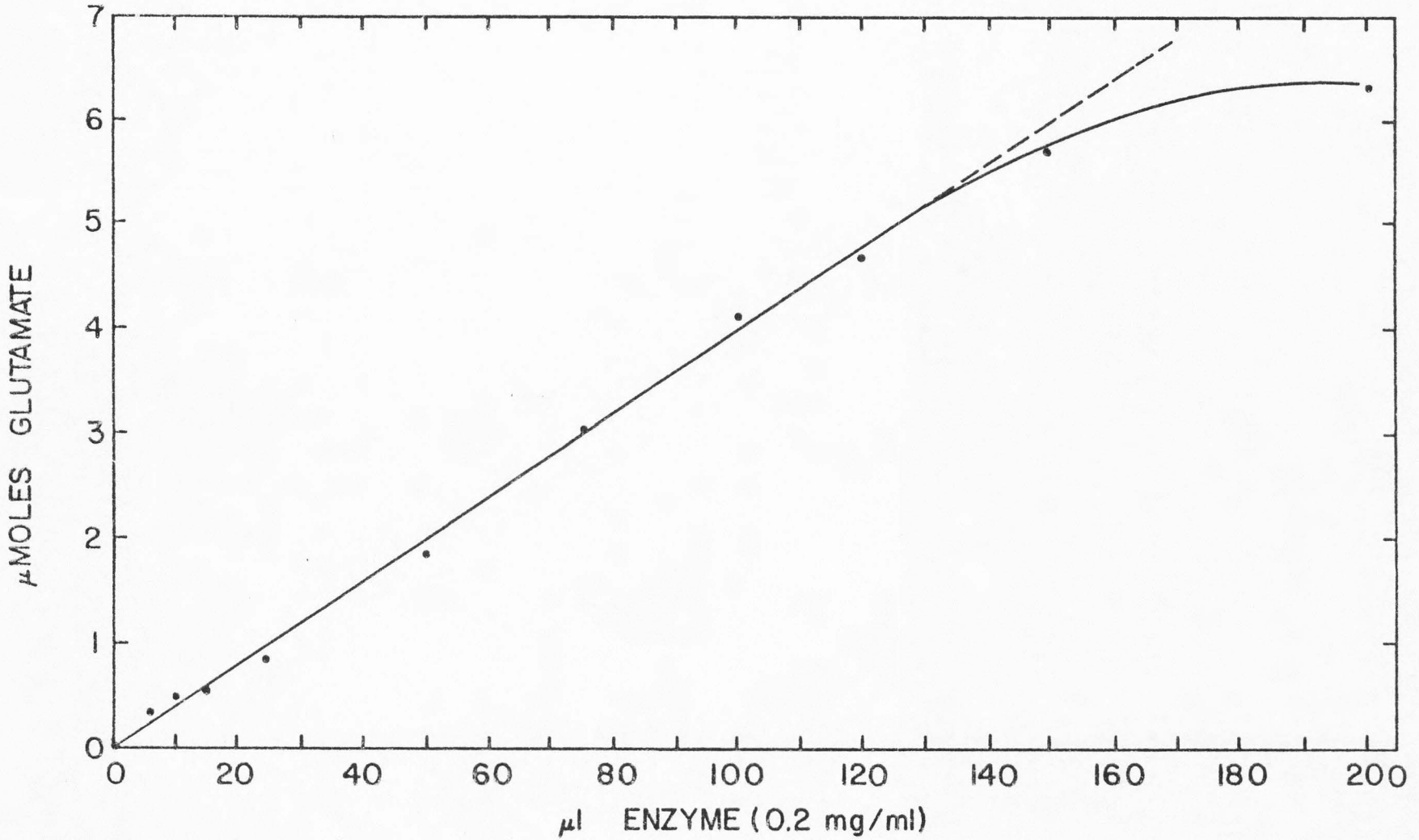

μMOLES GLUTAMATE
0
1
2
3
4
5
6
7
0
20
40
60
80
100
120
140
160
180
200
μl ENZYME (0.2 mg/ml)

Figure 5. Assay of commercial beef liver glutamate dehydrogenase using the coupled glutamate decarboxylase system. 5 μg of enzyme was used.

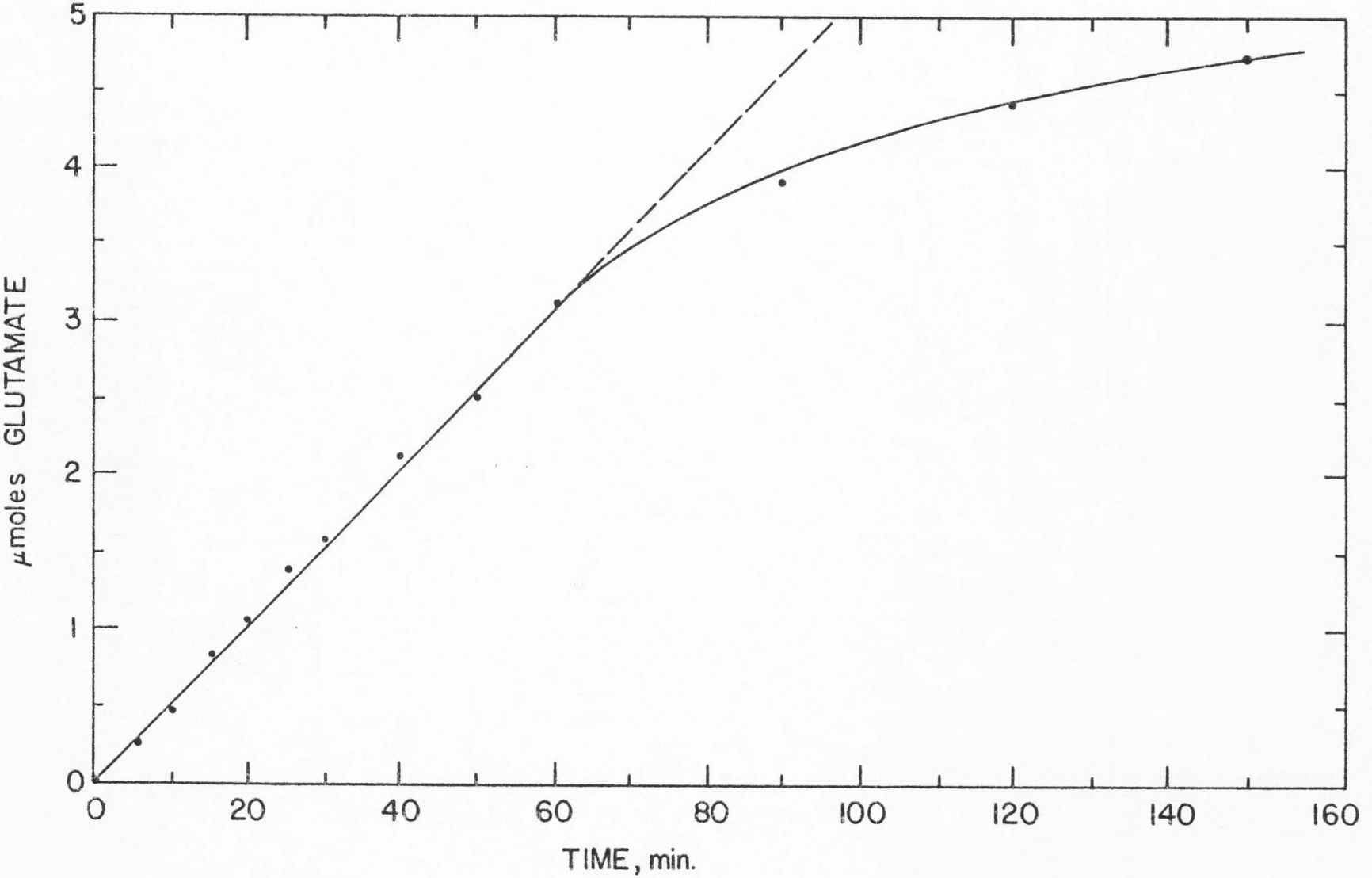

5
4
3
2
1
0
μmoles GLUTAMATE
0
20
40
60
80
100
120
140
160
TIME, min.

Figure 6. Assay of beef brain glutamate dehydrogenase using the coupled glutamate decarboxylase assay. One gram of brain was homogenized in 4 ml of 50 mM NaPi pH 7.7. 0.1 ml of extract was used per assay.

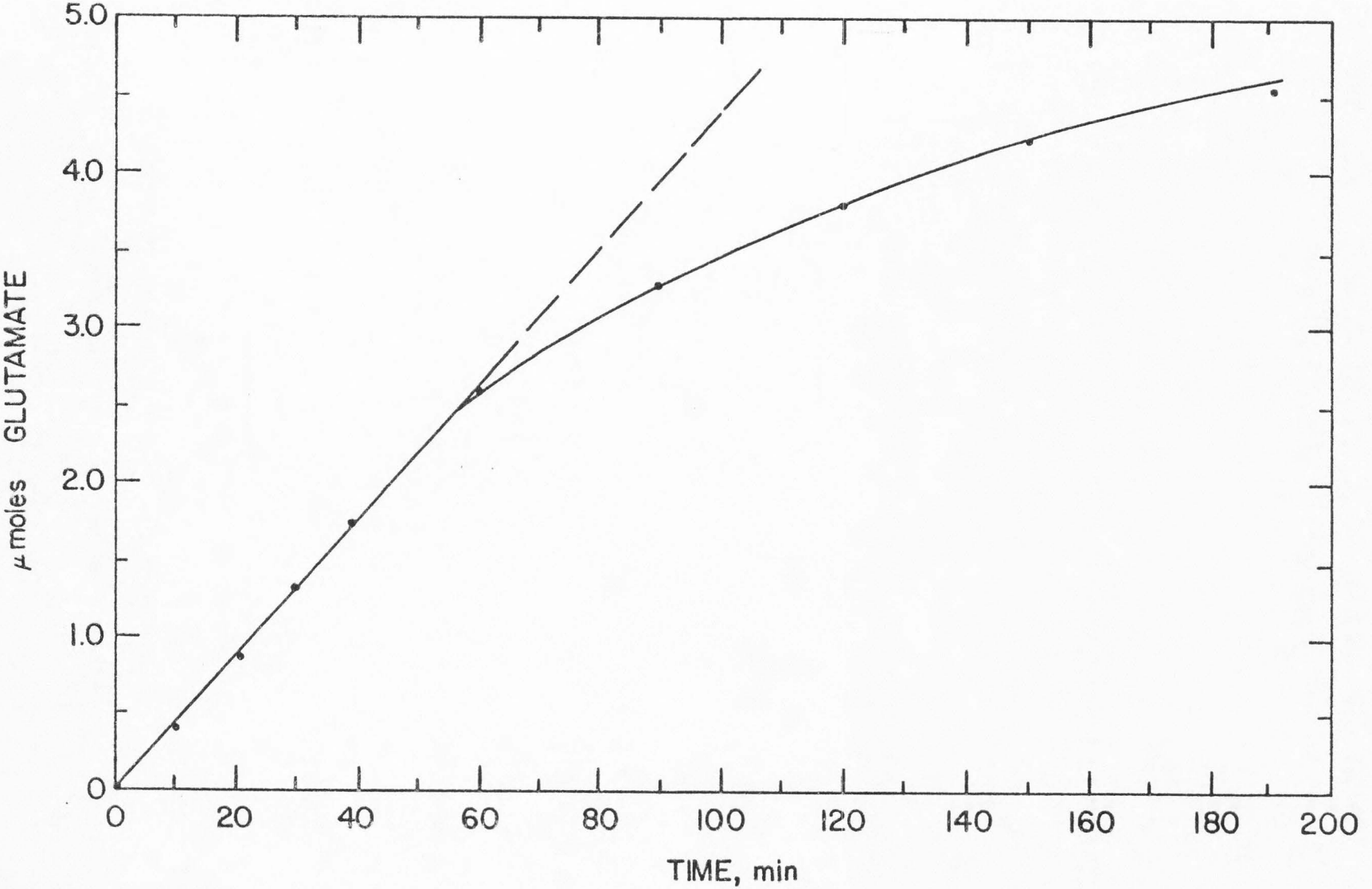

5.0
4.0
3.0
2.0
1.0
0
μ moles GLUTAMATE
0
20
40
60
80
100
120
140
160
180
200
TIME, min

Figure 7. Assay of beef brain glutaminase using the coupled glutamate decarboxylase system. One gram of brain was homogenized in 4 ml of borate buffer pH 8.0. 0.1 ml of extract was used per assay.

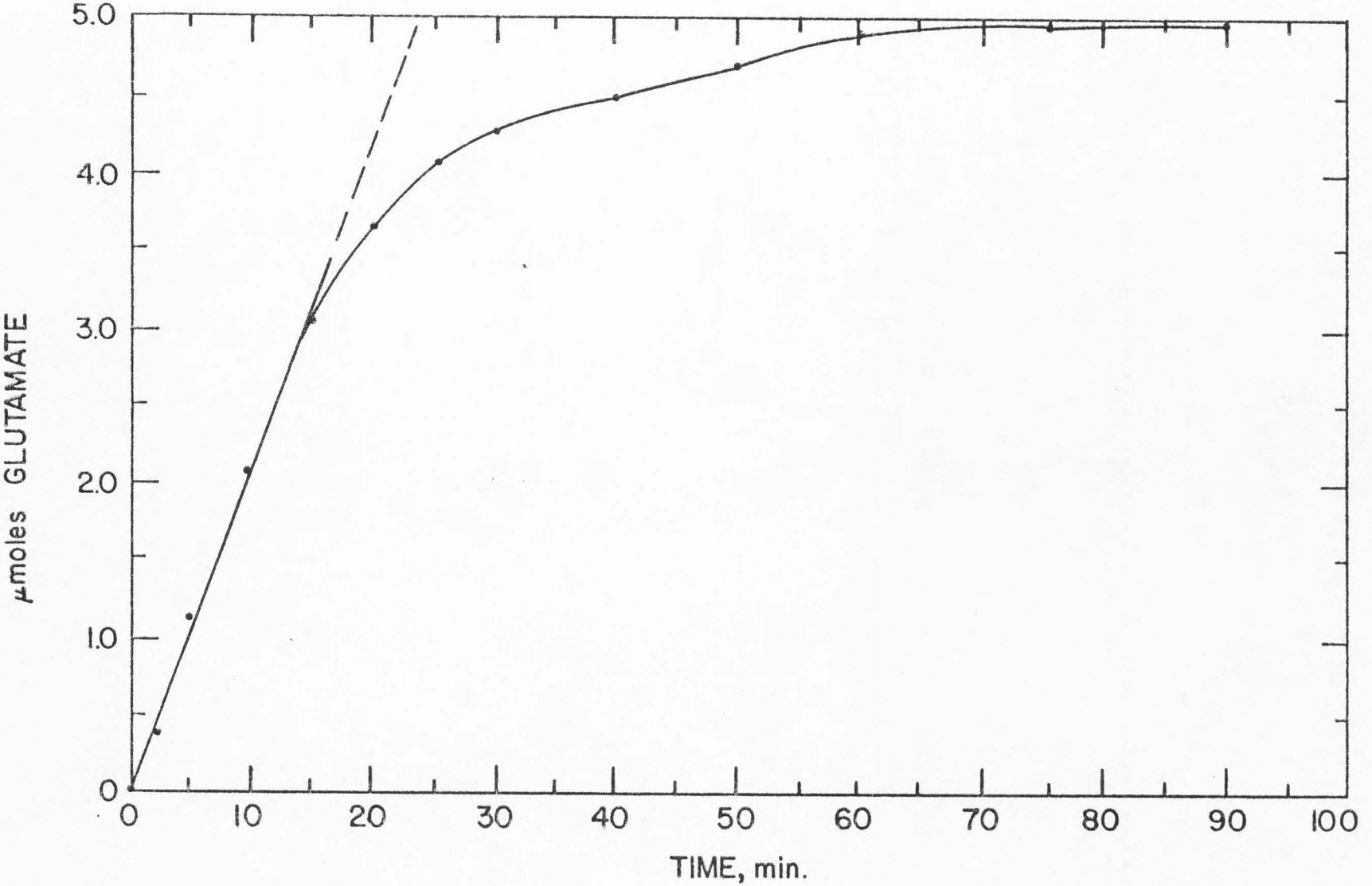

5.0
4.0
3.0
2.0
1.0
0
μmoles GLUTAMATE
0
10
20
30
40
50
60
70
80
90
100
TIME, min.

Figure 8. Assay of beef brain glutaminase using the coupled glutamate decarboxylase system. One gram of brain was homogenized in 4 ml of borate buffer pH 8.0. Time of assay was 15 minutes.

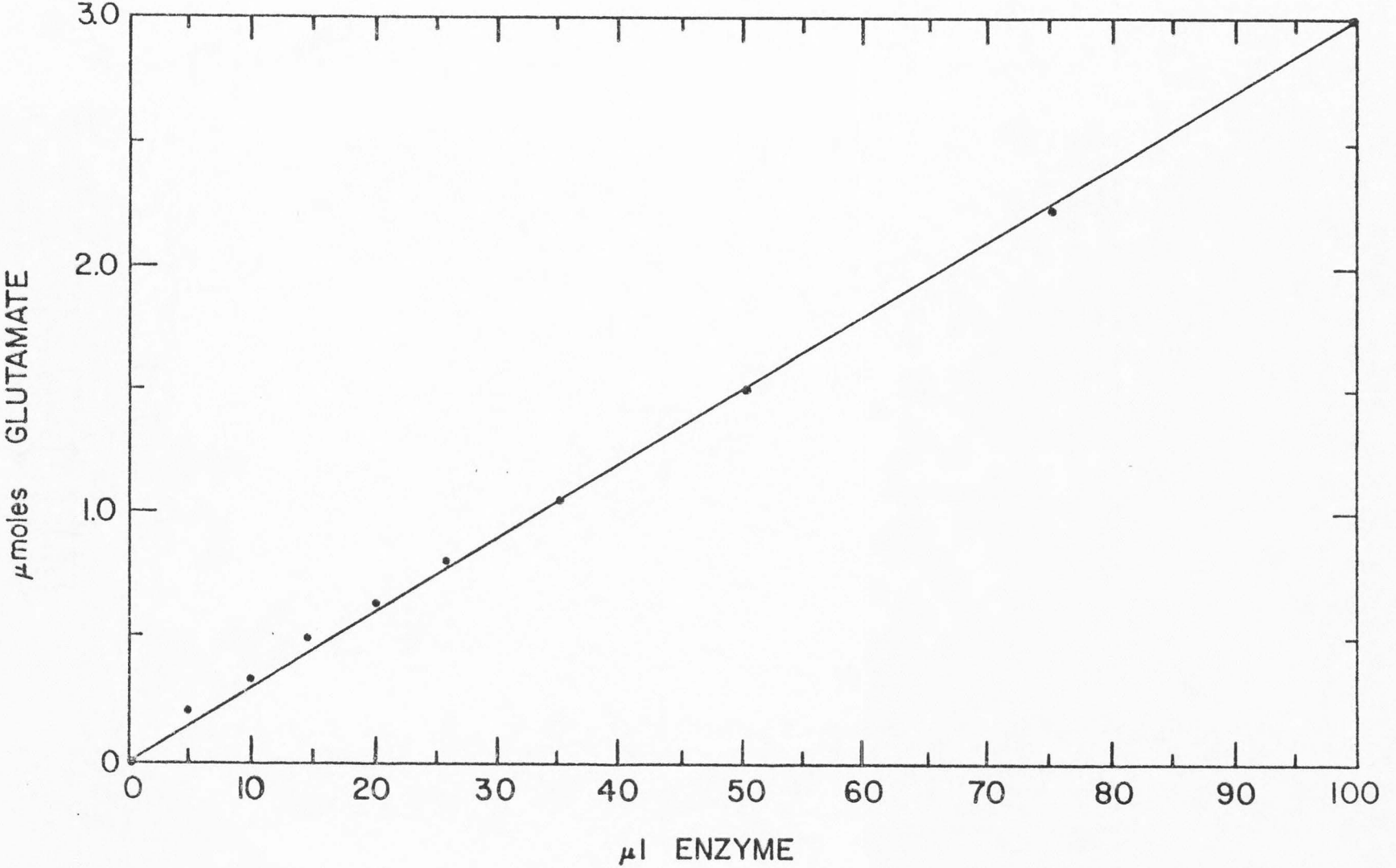

3.0
2.0
1.0
0
μmoles GLUTAMATE
0
10
20
30
40
50
60
70
80
90
100
μl ENZYME

Figure 9. Assay of commercial pig heart glutamate-oxaloacetate transaminase using the coupled glutamate decarboxylase system. Protein concentration was 8 μg/ml. Time was 15 minutes.

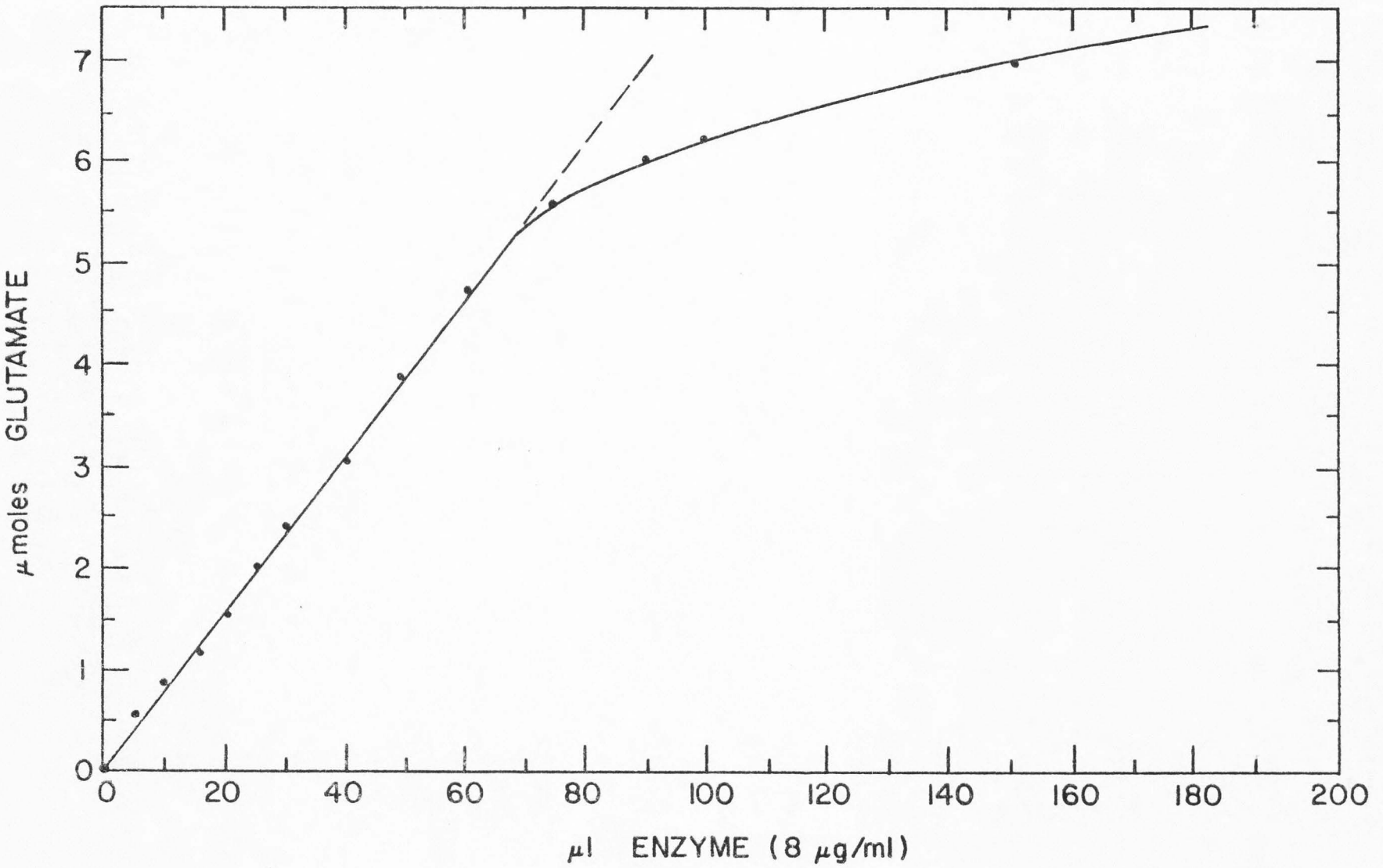

μmoles GLUTAMATE
0
1
2
3
4
5
6
7
0
20
40
60
80
100
120
140
160
180
200
μl ENZYME (8 μg/ml)

Figure 10. Assay of commercial pig heart glutamate-oxaloacetate transaminase using the coupled glutamate decarboxylase system. 0.08 µg of enzyme used.

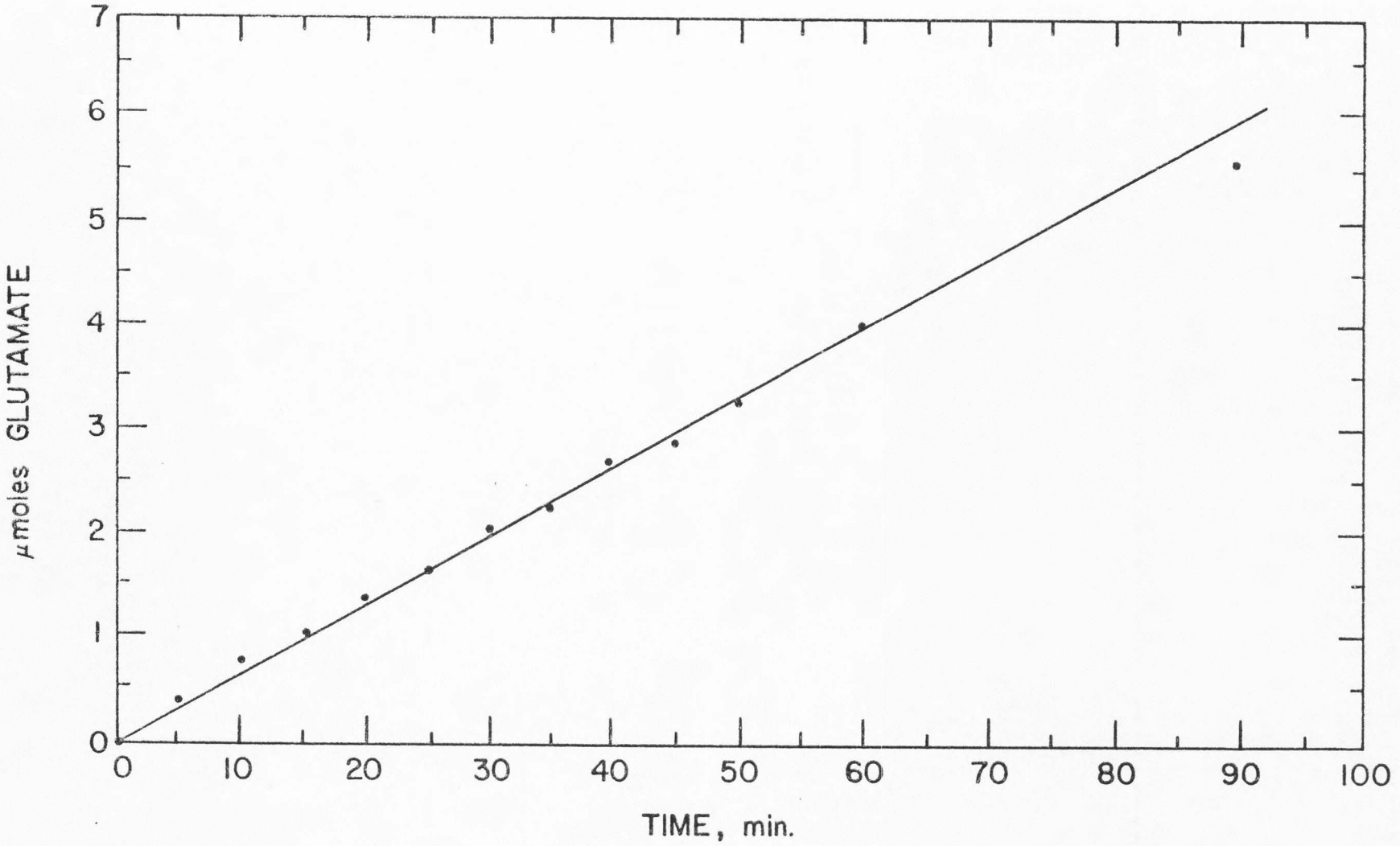

μmoles GLUTAMATE
0
1
2
3
4
5
6
7
0
10
20
30
40
50
60
70
80
90
100
TIME, min.

Figure 11. Assay of beef brain glutamate-oxaloacetate transaminase using the coupled glutamate decarboxylase system. One gram of brain was homogenized in 4 ml of NaPi buffer pH 7.7. Time of assay was 10 minutes.

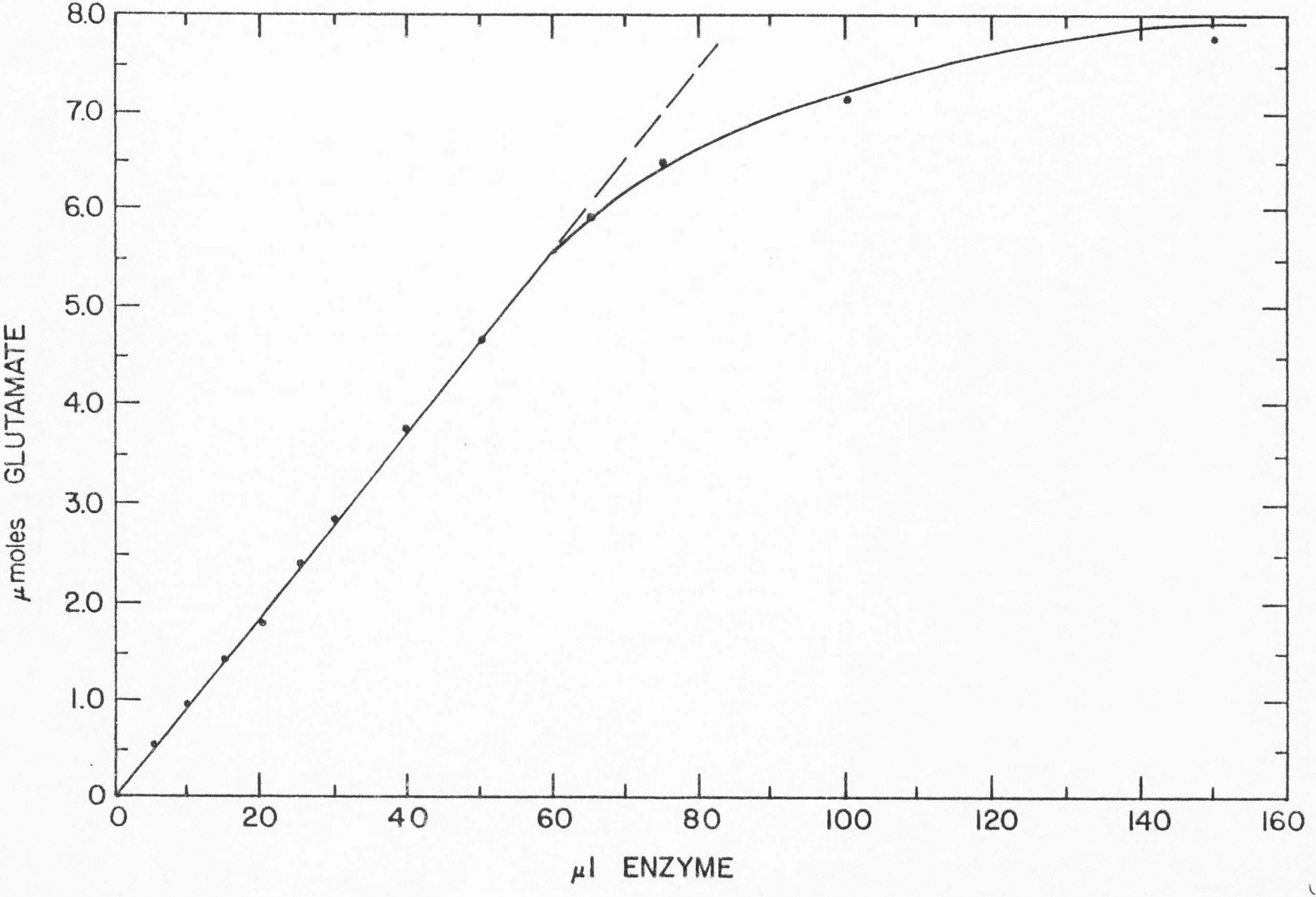

8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
μmoles GLUTAMATE
0
20
40
60
80
100
120
140
160
μl ENZYME

Figure 12. Assay of commercial pig heart glutamate-pyruvate transaminase using the coupled glutamate decarboxylase system. Enzyme concentration was 0.12 mg/ml. Time of assay was 15 minutes.

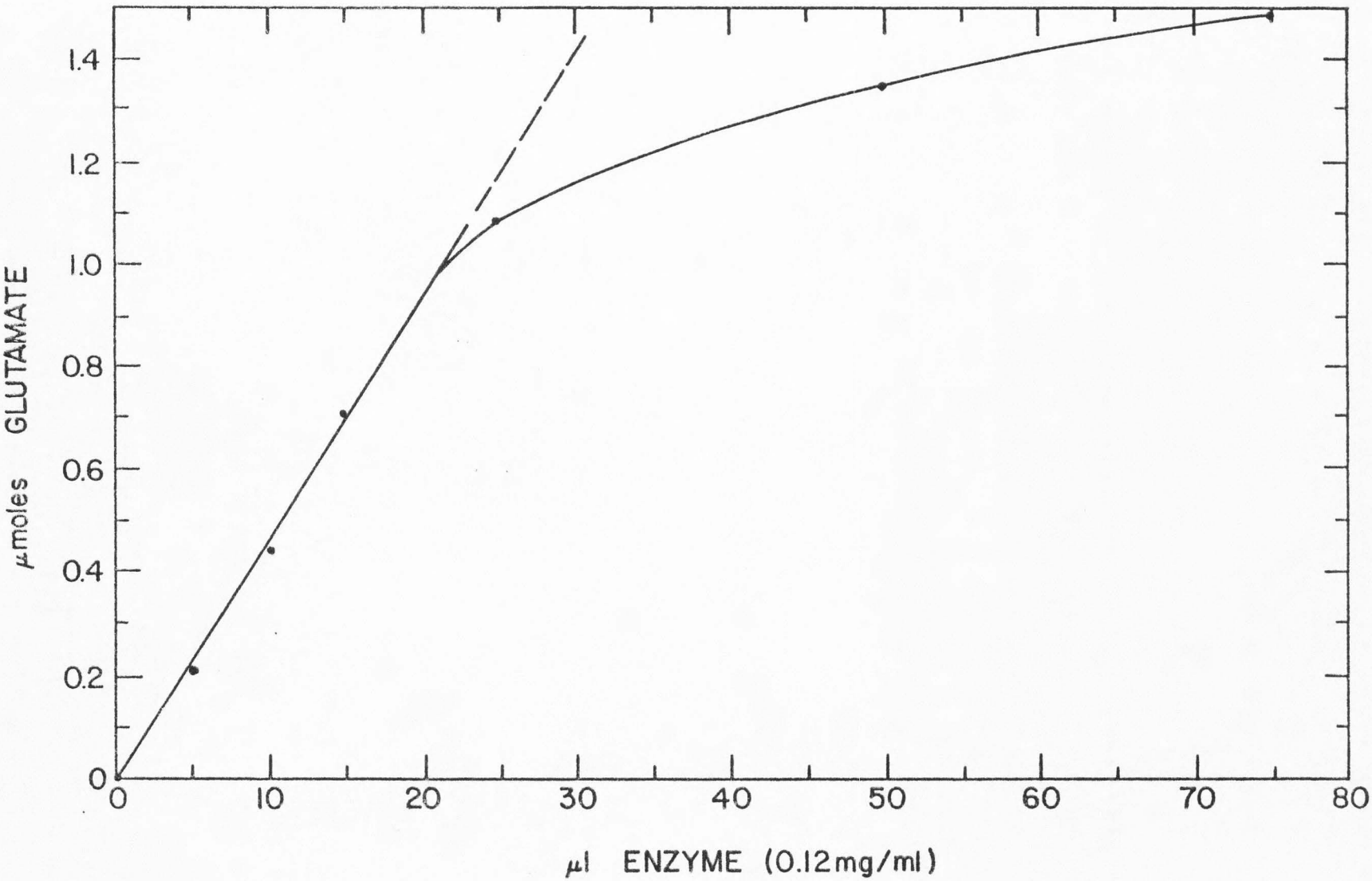

μmoles GLUTAMATE
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
70
80
μl ENZYME (0.12mg/ml)

Figure 13. Assay of commercial pig heart glutamate-pyruvate transaminase using the coupled glutamate decarboxylase system. Enzyme concentration was 0.024 mg/ml. Time of assay was 15 minutes.

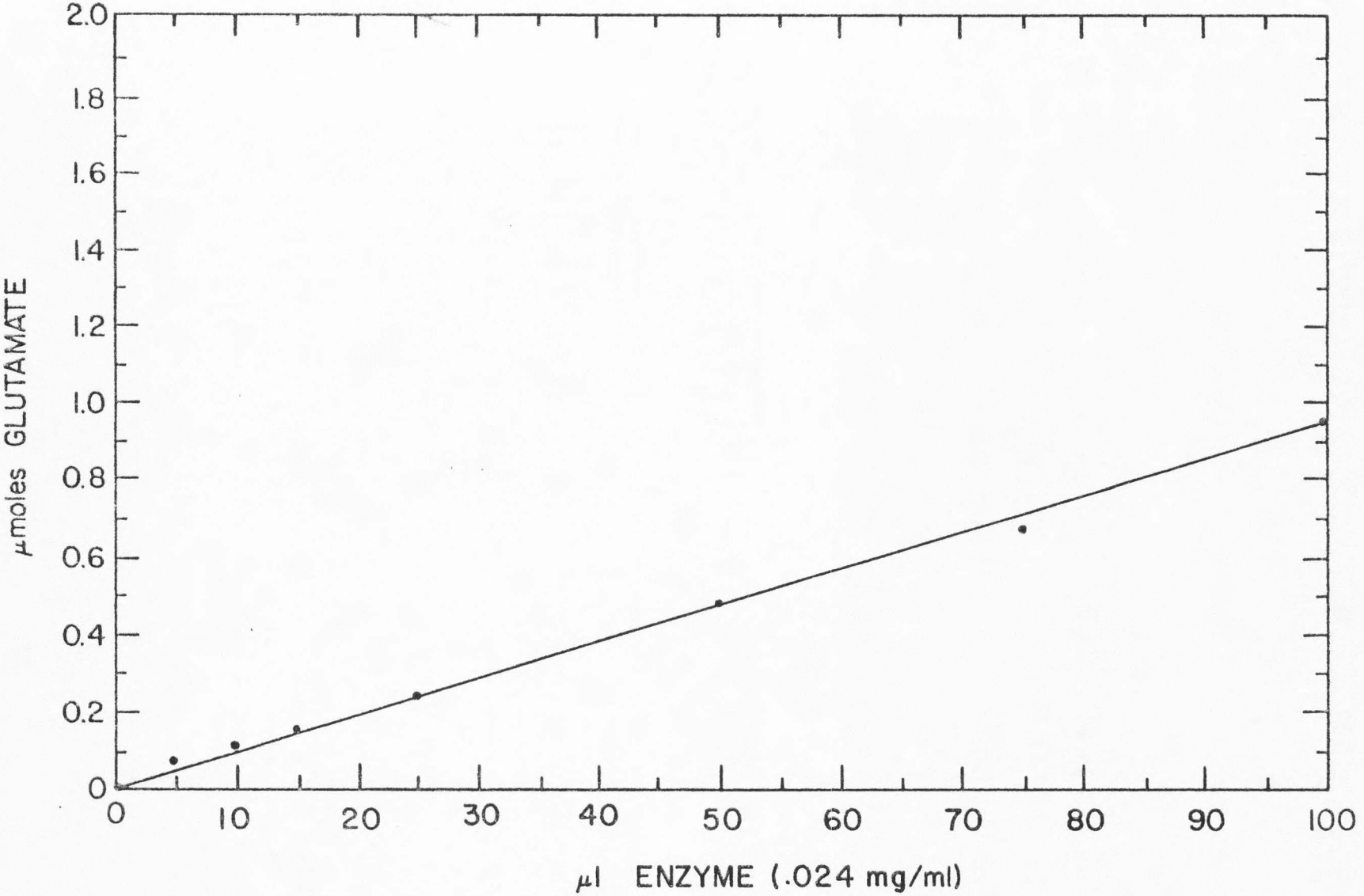

2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
μmoles GLUTAMATE
0
10
20
30
40
50
60
70
80
90
100
μl ENZYME (.024 mg/ml)

Figure 14. Assay of commercial pig heart glutamate-pyruvate transaminase using the coupled glutamate decarboxylase system. 0.6 μg enzyme was used.

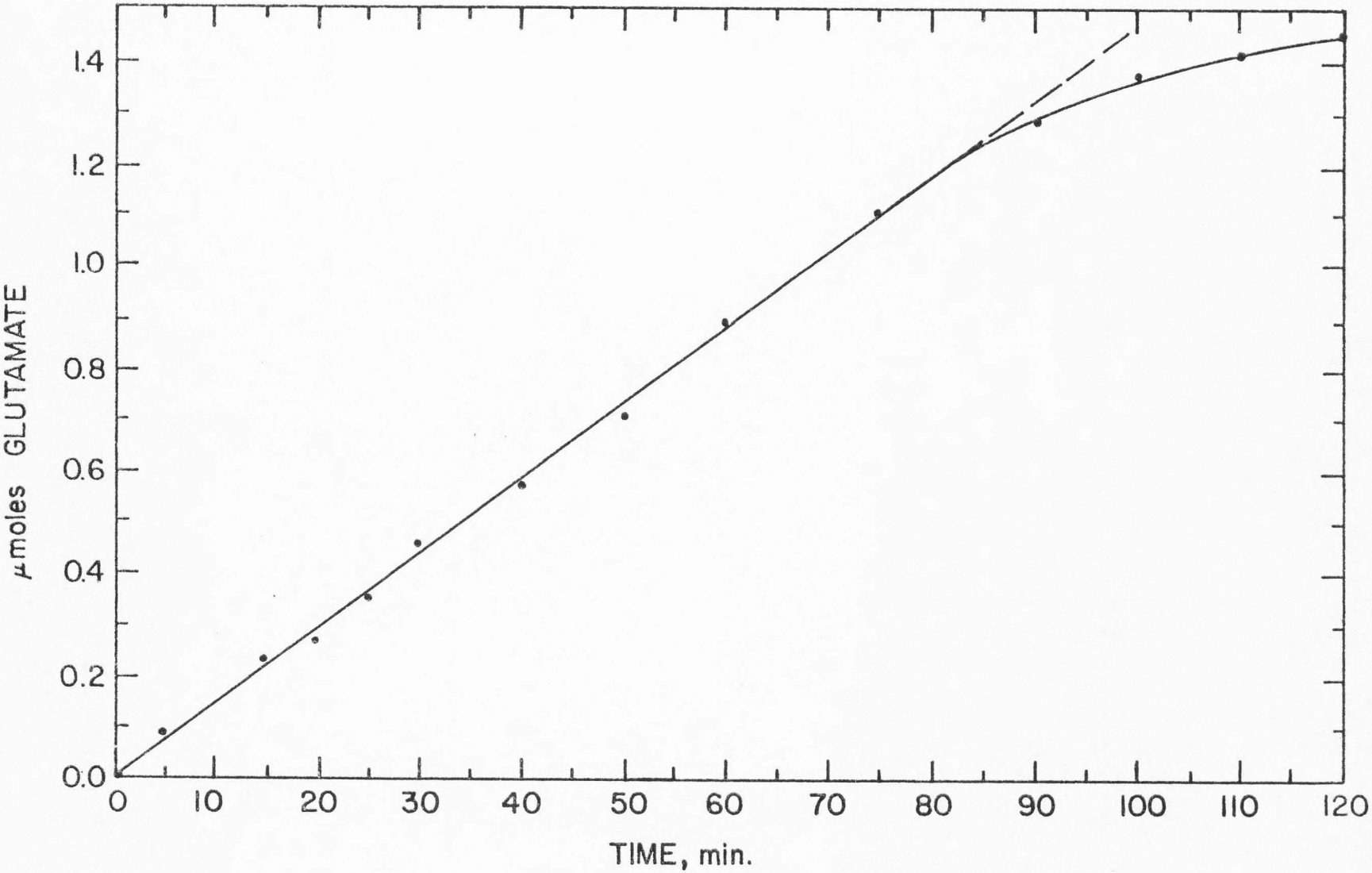

μmoles GLUTAMATE
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
70
80
90
100
110
120
TIME, min.

Figure 15. Assay of beef brain glutamate-pyruvate transaminase using the coupled glutamate decarboxylase system. One gram of brain was homogenized in 4 ml of Tris buffer pH 8.1. 0.15 ml of extract was used per assay.

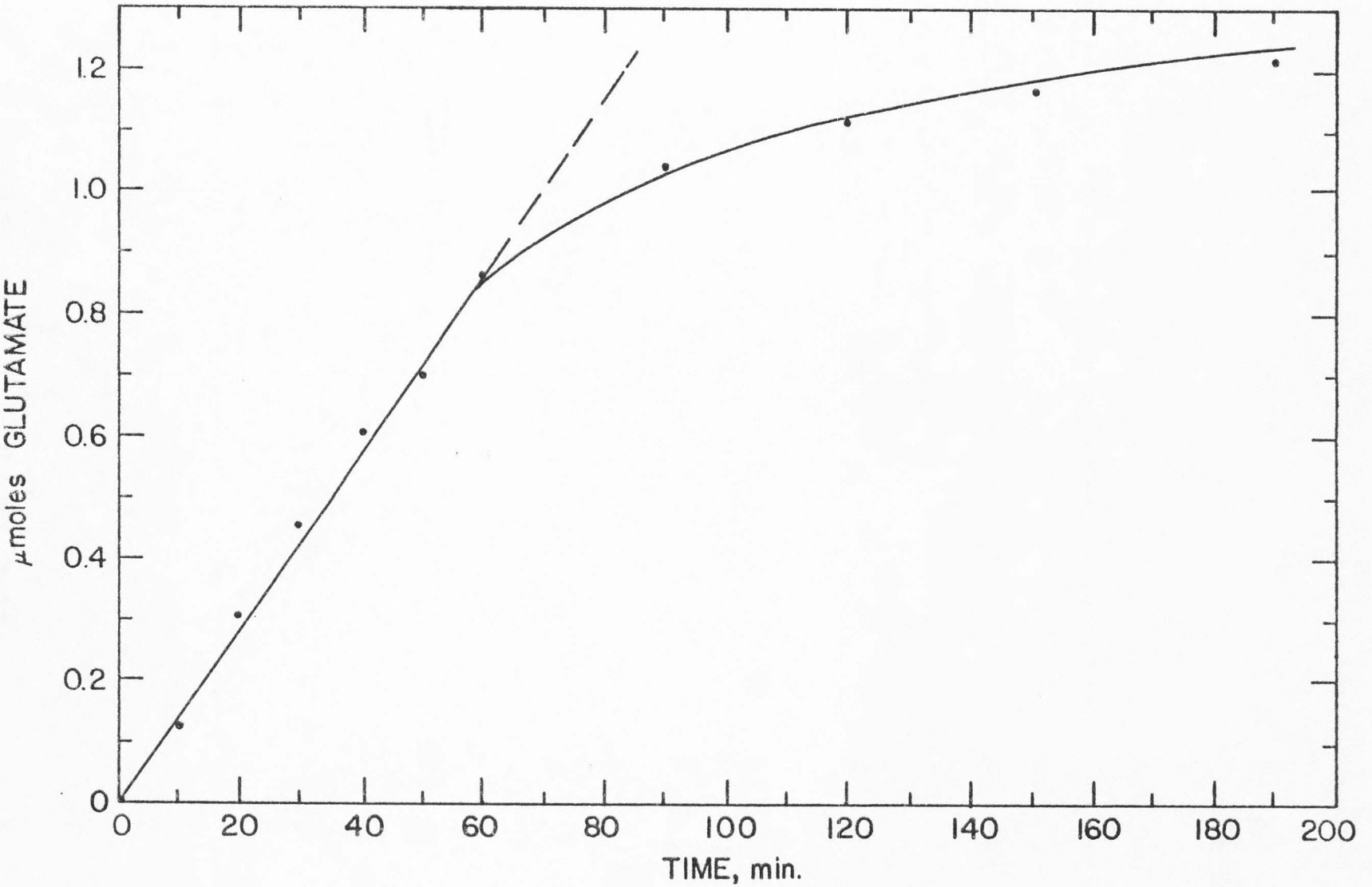

μmoles GLUTAMATE
1.2
1.0
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
120
140
160
180
200
TIME, min.

Figure 16. Assay of beef brain GABA-α-ketoglutarate transaminase using the coupled glutamate decarboxylase system. One gram of brain was homogenized in 4 ml of Tris buffer pH 8.2. Time of assay was 15 minutes.

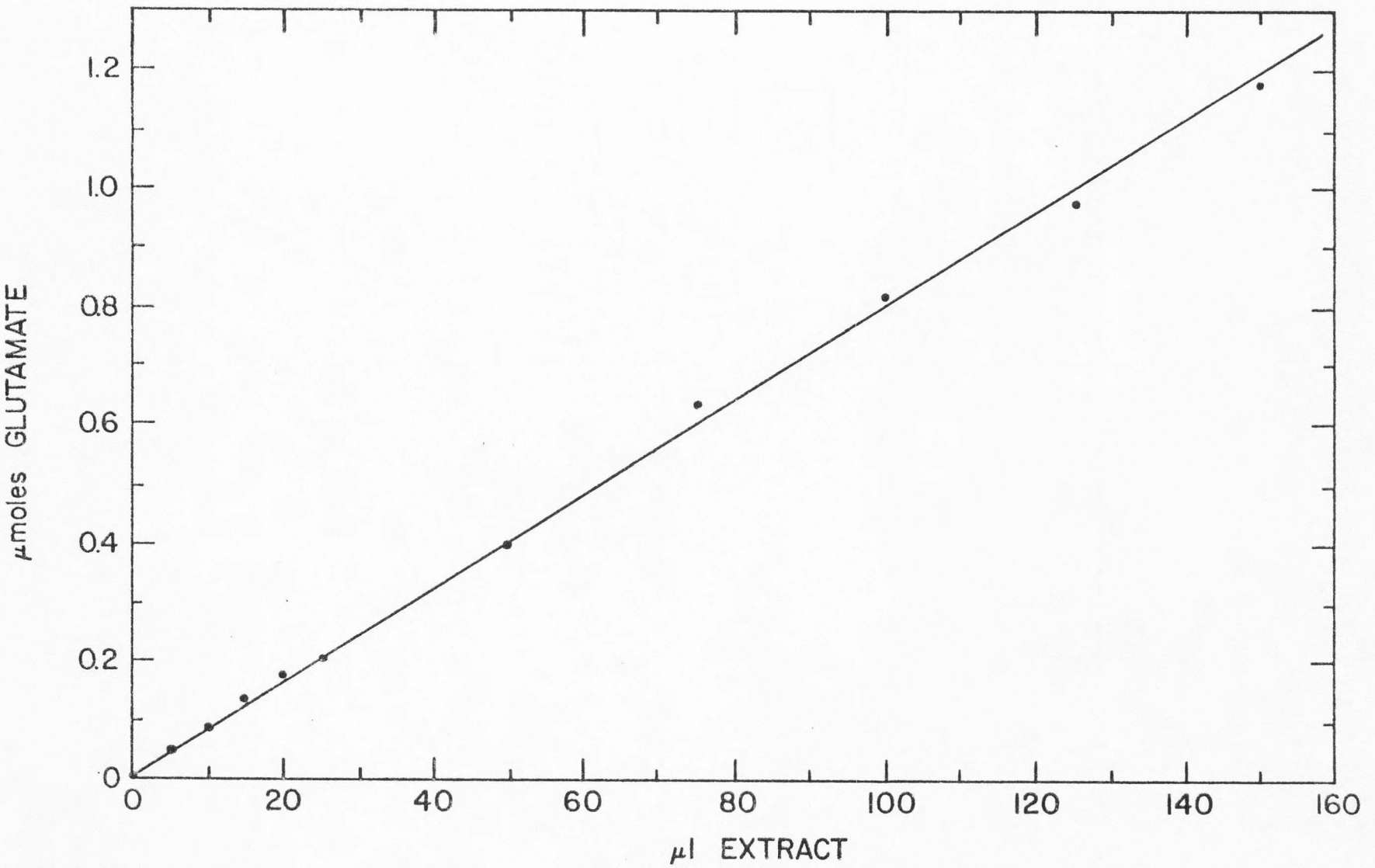
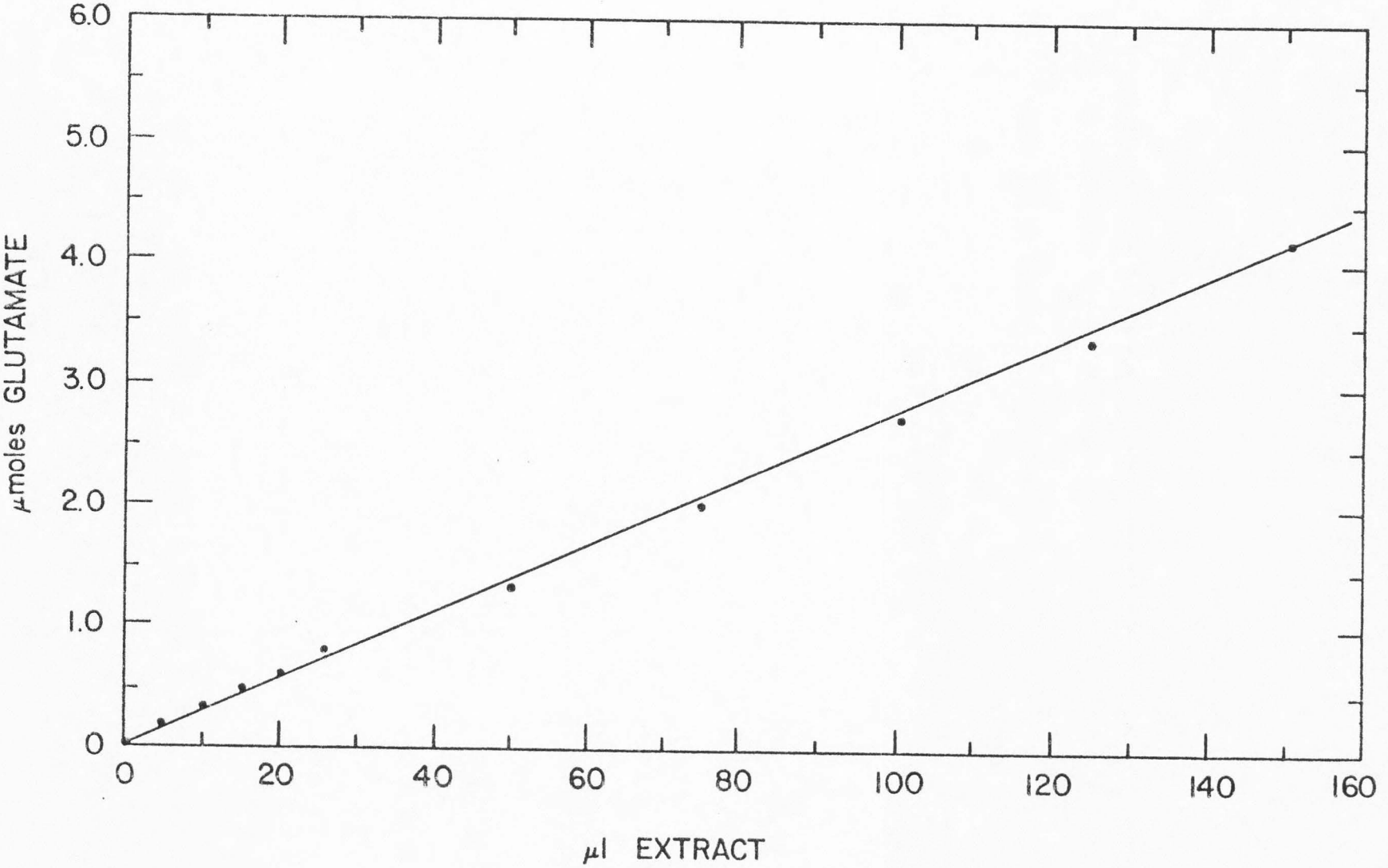

μmoles GLUTAMATE
1.2
1.0
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
120
140
160
μl EXTRACT

Figure 17. Assay of beef brain GABA-α- ketoglutarate transaminase using the coupled glutamate decarboxylase system. One gram of brain was homogenized in 4 ml of Tris buffer pH 8.2. Time of assay was 1 hour.


6.0
5.0
4.0
3.0
2.0
1.0
0
μmoles GLUTAMATE
0
20
40
60
80
100
120
140
160
μl EXTRACT

extracts of frozen beef brain which showed that this assay system is suitable for the determination of glutamate-pyruvate transaminase.

Figures 16 and 17 give the results of the coupled assays for GABA-α-ketoglutarate transaminase. Since no commercial preparations of this enzyme were available, the assays were done with only the extracts of frozen beef brain. Because activities of this enzyme seemed to be low, the assays were linear for long periods of time, and the point at which deviation from linearity began was difficult to determine.

No attempt was made to assay for brain glutamate decarboxylase.

Table 9 compares the sensitivity and costs for some of the different assays that are available for glutamate producing enzymes. The costs for the spectrophotometric assays are based on 1 ml reaction volumes and the substrate concentrations specified in tables 1 to 5. Costs for NAD^+ and NADH were taken from the current Sigma catalog. The costs and sensitivities for the radiometric assays are based on 0.4 ml reaction volumes and the substrate concentrations also specified in tables 1 to 5. At these levels the radioisotope adds only 10 cents to the cost of each assay, based on current prices from New England Nuclear.

Spectrophotometric assays based on the absorbance of NAD^+ and NADH are cheap and can be made fairly sensitive,

Table 9. Comparison of the Sensitivities and Costs of Some Different Assays Available For The Enzymes of the Glutamate-GABA Systen

Enzyme	Type of Assay (substance detected)	Sensitivity[a] (nanomoles)	Cost/ Assay	References
Glutamate Dehydrogenase	Spectrophotometric (NADH)	16	5¢	(Horecker, B.L., 1948)
Glutaminase	Spectrophotometric (NAD^+)	16	15¢	(Segel, I.H., 1968)
Glutamate-Oxaloacetate Transaminase	Colorimetric (DTNB)	7	-	(Itoh and Srere, 1970)
Glutamate-Oxaloacetate Transaminase	Spectrophotometric (OAA)	83	-	(Kornberg, et al., 1948)
Glutamate-Pyruvate Transaminase	Radiometric 1-(^{14}C)-α-ketoglutarate	7	10¢	

a. For spectrophotometric assays, the calculations are based on a minimum absorbance change of 0.1 and an assay volume of 1 ml. For the radiometric assays, an assay volume of 0.4 ml and a mimimum change of 400 DPM were used. A substrate concentraction of 10 X K_m was used in each case.

but they are useless in crude tissue extracts where other dehydrogenases interfere. In addition, when enzyme levels are low and it is necessary to add large amounts of a crude extract to an assay, nucleic acids and proteins may absorb enough at 340 nm to interfere with this assay. Some of the other colorimetric assays, such as the DTNB assay for glutamate-oxaloacetate transaminase, are about equally sensitive, but can be used for only one particular enzyme.

These radiometric techniques can provide sensitivities 100 to 1000 times higher than most other assays that are available, although the cost for maximum sensitivity would be prohibitive. This cost can be reduced considerably, if necessary, by performing the assays at less than saturating substrate concentrations and by reducing total volumes. Where enzyme activities are low, the radiometric assay will be the method of choice.

In conclusion, it appears that to use a different type of assay system for each enzyme that will be measured would result in a set of four or five different assays that, at best, would be tedious and difficult to perform. Since each assay would present a varying degree of sensitivity, this approach may lead to confusing and possibly erroneous results concerning the regulation of the enzymes of the GABA-glutamate system. Clearly, the radiometric techniques presented here can overcome these problems by unifying these varied assays into one simple, sensitive method.

SUMMARY

This thesis is an attempt to lay the groundwork for the larger goal of understanding the regulation of GABA and glutamate concentrations in brain and nerve tissue, which, in turn, may help to understand the control of the nerve impulse. This work has established a convenient, accurate, and sensitive assay system for the enzymes controlling GABA and glutamate metabolism, using a coupled, radiometric assay employing glutamate decarboxylase.

The glutamate decarboxylase was obtained from a commercial acetone powder by simplifying existing procedures. The enzyme preparation appeared to be at least 95% pure as judged by gel electrophoresis in 0.1% sodium dodecyl sulfate. The enzyme had a specific activity that was routinely between 41 and 72 units/mg. It was judged sufficiently pure to be used in the coupled enzyme assays.

The coupled enzyme assays were checked with commercial preparations of each enzyme, where available, and with crude beef brain homogenates. All of the assays were shown to be linear with respect to both time and enzyme concentration, thus assuring the feasibility of the procedure.

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